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HIGHLY SENSITIVE FLUORIMETRIC METHOD FOR DETERMINATION OF VARENICLINE IN ITS BULK AND TABLETS VIA DERIVATIZATION WITH 7-CHLORO-4-NITROBENZOXADIAZOLE

M. G. KASSEM[a], I. A. DARWISH[a*], H. W. DARWISH[a, b]
[a] *Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.*
[b] *Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., Cairo 11562, Egypt.*

This study represents the first report on the development and validation of a highly sensitive fluorimetric method for determination of varenicline (VRC) in tablets and plasma. The method was based on nucleophilic substitution reaction of VRC with 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) in an alkaline buffered medium (pH 9) to form a highly fluorescent derivative that exhibited maximum fluorescence intensity at 550 nm after excitation at 470 nm. The factors affecting the reaction were carefully optimized. The stoichiometry of the reaction was determined, and the mechanism was postulated. Under the optimum reaction conditions, a linear relationship with good correlation coefficient (r = 0.9993) was found between the fluorescence intensity and VRC concentrations in the range of 5-250 ng ml^{-1}. The limits of detection and quantitation were 2.5 and 8.3 ng ml^{-1}, respectively. The method was reproducible as the relative standard deviations of the results did not exceed 2%. The proposed method was successfully applied to the determination of VRC in its bulk and tablets with good accuracy; the label claim percentage was 99.17 ± 1.06%. The proposed method is valuable for routine application in quality control laboratories for determination of VRC.

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1. Introduction

Varenicline (VRC) is a novel agent that is a centrally acting as a highly selective partial agonist for the nicotinic acetylcholine receptor [1]. VRC has mixed agonistic-antagonistic properties, thus it has the therapeutic benefit of relieving the symptoms of nicotine withdrawal and cigarette craving during abstinence while blocking the reinforcing effect of nicotine in those who lapse [2-4]. VRC tartrate has been approved by the USA-FDA as an aid to smoking cessation [5]. The approved regime of VRC is 1 mg for 12 weeks, starting with a one-week titration period [6].

The effective and safe therapy with VRC is basically depending on the quality of its pharmaceutical preparations (tablets), and assessing its concentrations in tablets for the purposes of quality control. As well, the therapeutic benefits profile of VRC is anticipated to encourage the development of new pharmaceutical preparations for VRC. As a consequence, there is an increasing demand for a proper analytical method for determination of VRC in its bulk drug and finished pharmaceutical formulations.

VRC has not yet been officially described in any pharmacopoeia. In literature, few methods have been found describing the quality control of VRC [7-11]. However, these methods involved complex and expensive instrumentation such as ultra-pressure liquid chromatography (UPLC) [8], LC-tandem spectrometry [9] and capillary electrophoresis [10].

*Corresponding author: idarwish@ksu.edu.sa

Fluorimetry is considered one of the most convenient analytical techniques, because of its inherent simplicity, high sensitivity, low cost, and wide availability in most quality control and clinical laboratories. No attempt has yet been made for the fluorimetric determination of VRC. The present study describes, for the first time, the development of a highly sensitive and simple fluorimetric method for the determination of VRC in its bulk and tablets. The method was based on the reaction of VRC with 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) in alkaline buffered medium to produce a highly fluorescent product that was measured fluorimetrically at 550 nm after excitation at 70 nm.

2. Experimental

Apparatus

FP-6200 fluorimeter (JASCO Co. Ltd., Kyoto, Japan), with 1-cm quartz cells was used for all measurements. The slit width of both the excitation and emission monochromators was set at 1.5 nm. The calibration and linearity of the instrument were frequently checked with standard quinine sulphate (0.01 μg ml^{-1}). Wavelength calibration was performed by measuring $\lambda_{excitation}$ 275 nm and $\lambda_{emission}$ 430 nm; no variation in the wavelength was observed. pH meter, Model 350 (Bibby Scientific Ltd., T/As Jenway, Essex, England). MLW type thermostatically controlled water bath (Memmert GmbH, Co. Schwa bach, Germany).

Reagents and Materials

Varenicline tartarate standard with claimed purity of 99.6% was purchased from Weihua Pharma Co. Ltd. (Zhejiang, China). A solution of 0.2% (w/v) of NBD-Cl (Sigma Chemical Co., St. Louis, USA) was freshly prepared by dissolving 100 mg in 50 ml acetone and protected from light during use. Champix® tablets (Pfizer Inc, New York, USA), labeled to contain 1 mg VRC per tablet was obtained from the local market. Double distilled water was obtained through WSC-85 water purification system (Hamilton Laboratory Glass Ltd., Kent, USA) and used throughout the work. All solvents and materials used throughout this study were of analytical grade.

Preparation of solutions

Standard VRC solution

An accurately weighed amount (10 mg) of VRC was quantitatively transferred into a 10-ml calibrated flask, dissolved in 10 ml distilled water, to obtain a stock solution of 1 mg ml^{-1}. The stock solution was found to be stable for at least four weeks when kept in refrigerator. This stock solution was further diluted with water to obtain working solutions in the range of 5–250 ng ml^{-1}.

Tablets sample solutions

Twenty tablets were weighed and finely powdered. An accurately weighed quantity of the powder equivalent to 10 mg of the active ingredient was transferred into a 10-ml calibrated flask, and dissolved in about 5 ml of distilled water. The contents of the flask were swirled, sonicated for 5 min, and then completed to volume with water. The contents were mixed well and filtered; the first portion of the filtrate was rejected. The filtered solution was diluted quantitatively with distilled water to obtain suitable concentrations for the analysis.

Buffer solutions

Clark and Lubs buffer solution was prepared by mixing 50 ml of 0.2 M aqueous solutions of boric acid and potassium chloride (1 liter containing 12.368 g of boric acid and 14.90 g of potassium chloride) with 21.3 ml of 0.2 M sodium hydroxide in 200 ml standard flask [12], and adjusted by pH meter. Tris buffer was prepared by mixing 100 ml 0.1 M tris(hydroxymethyl)aminomethane with 29.4 ml of 0.1 M HCl [13]. Britton-Robinson buffer composed of 0.04 M boric acid, 0.04 M phosphoric acid and 0.04 M acetic acid adjusted at pH 9 by using 0.2 M sodium hydroxide [13]. Phosphate buffer composed of 0.1 M disodium hydrogen phosphate (14.2 g l^{-1}) and 0.1 M sodium hydroxide [13].

General recommended procedures

One milliliter of VRC solution containing 5–250 ng ml^{-1} was transferred into separate 10-ml calibrated flask. A 1 ml of Clark buffer solution of pH 9 and 1 ml of NBD-Cl solution (0.2%, w/v) were added. The reaction solutions were allowed to proceed for 20 min in thermostatically controlled water bath at 60 ± 5 ºC. The solutions were left for cooling at room temperature 25 ± 2 ºC, acidified by adding 1 ml of HCl (25 mM), and completed to volume with water. The fluorescence intensity of the resulting solution was measured at 550 nm after excitation at 470 nm against reagent blanks prepared in the same manner with 1 ml water instead of the sample solution.

Determination of the stoichiometric ratio of the reaction

Limiting logarithmic method [14] was employed. Two sets of experiments were carried out employing the general recommended procedures described above. The first set of experiments were carried using increasing NBD-Cl concentrations (5×10^{-3} – 2×10^{-2} M) at fixed VRC concentration (2×10^{-4} M). The second set of experiments were carried using increasing VRC concentrations (1×10^{-4} – 1×10^{-3} M) at fixed NBD-Cl concentration (1×10^{-3} M). The logarithms of the obtained fluorescence intensities were plotted as a function of the logarithms of the NBD-Cl and VRC concentration in the first and second sets of experiments, respectively. The slopes of the fitting lines in both sets of experiments were calculated.

3. Results and discussion

Because of the absence of native fluorescence of VRC, its derivatization with fluorigenic reagent was necessary for its fluorimetric determination. NBD-Cl was chosen as a derivatizing reagent because it forms highly fluorescent derivatives with amines using relatively mild reaction conditions [15]. Owing to the presence of labile chloride in the chemical structure of NBD-Cl, a daily fresh solution was prepared and tested in the present study. It was found that VRC reacted with NBD-Cl and formed a golden yellow fluorescent derivative. This derivative exhibited maximum fluorescence intensity (λ_{em}) at 550 nm after its excitation at wavelength (λ_{ex}) of 470 nm. The excitation and emission spectra for the reaction product of VRC with NBD-Cl are given in Fig. 1.

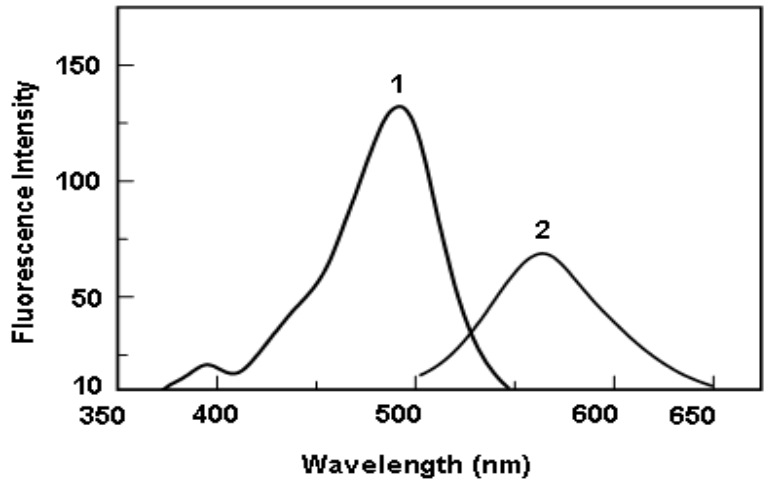

Fig. 1. Excitation (1) and emission (2) spectra of the reaction product of VRC (50 ng/mL) with NBD-Cl (0.2%, w/v).

Optimization of reaction variables

Effect of NBD-Cl concentration

Studying the effect of NBD-Cl concentration on its reaction with VRC revealed that the reaction was dependent on NBD-Cl concentration as the fluorescence intensity (FI) of the reaction solution increased as the NBD-Cl concentration increased (Fig. 2). The highest readings were attained at NBD-Cl concentration of 0.2% (w/v), beyond which the FI slightly decreased. Therefore, the subsequent experiments were carried out using 0.2%.

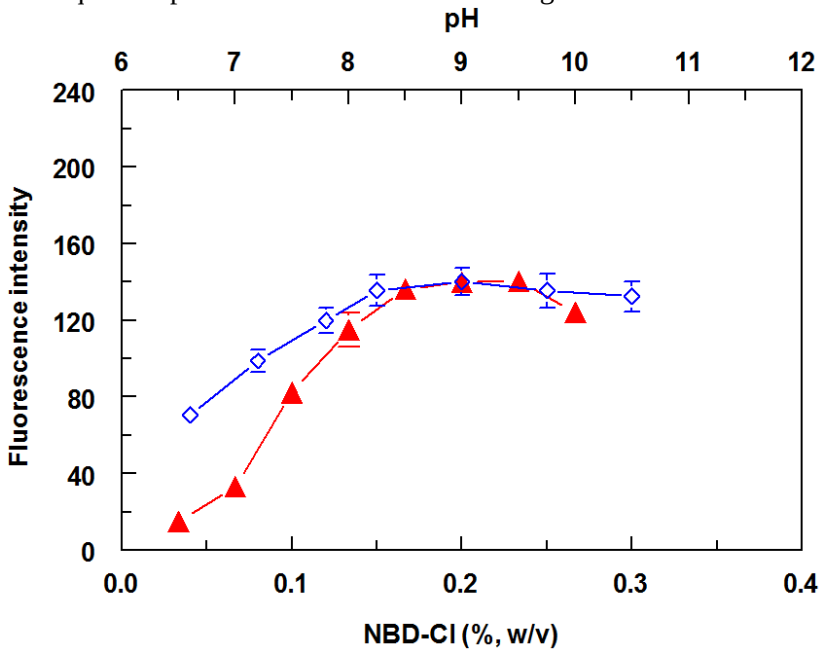

Fig. 2. Effect of NBD-Cl concentration (▲) and pH (◇) on the reaction of VRC (80 ng/mL) with NBD-Cl (0.2%, w/v).

Effect of pH and buffer component

In order to generate the nucleophile from VRC, being as acid salt (tartarate), it was anticipated that the reaction should be carried out in alkaline medium. The influence of pH on the reaction of VRC with NBD-Cl was investigated by carrying out the reaction in buffered solution at varying pH values. The results revealed that VRC had difficulty to react with NBD-Cl in acidic media (Fig. 2). This was attributed to the existence of the amino group of VRC in the form of salt, thus it loses its nucleophilic substitution capability. As the pH increased the readings increased rapidly, as the amino group of VRC turned into the free form, thus facilitating the nucleophilic substitution. The maximum readings were attained at pH values of 8.5–9.5. Subsequent experiments were carried out at pH 9.

In order to investigate the effect of buffer components on the reaction, different buffer solutions of pH 9 were tested: Clark, Robinson, phosphate, borate, carbonate and tris buffers. The highest fluorescence intensity were obtained when Clark buffer was used, thus it was used in all the subsequent experiments.

Effect of temperature and time

In order to determine the optimum temperature required for the reaction, it was carried out at room temperature (25 ± 5 °C) and at elevated temperatures (40–80 °C) and the induced FI values were measured. It was found that the reaction was dependent on the temperature as the FI increased as the temperature increased and the maximum FI was obtained at 60 °C, beyond which the FI decreased (Fig. 3). This observation was coincident with the results that have been previously reported by Aktas E.S. *et al* [13]. Therefore, subsequent investigations were carried out at 60 °C.

In order to determine the time required for completion of the reaction, the induced fluorescence was monitored for 30 min. The results indicated that the optimum time was 20 min (Fig. 3).

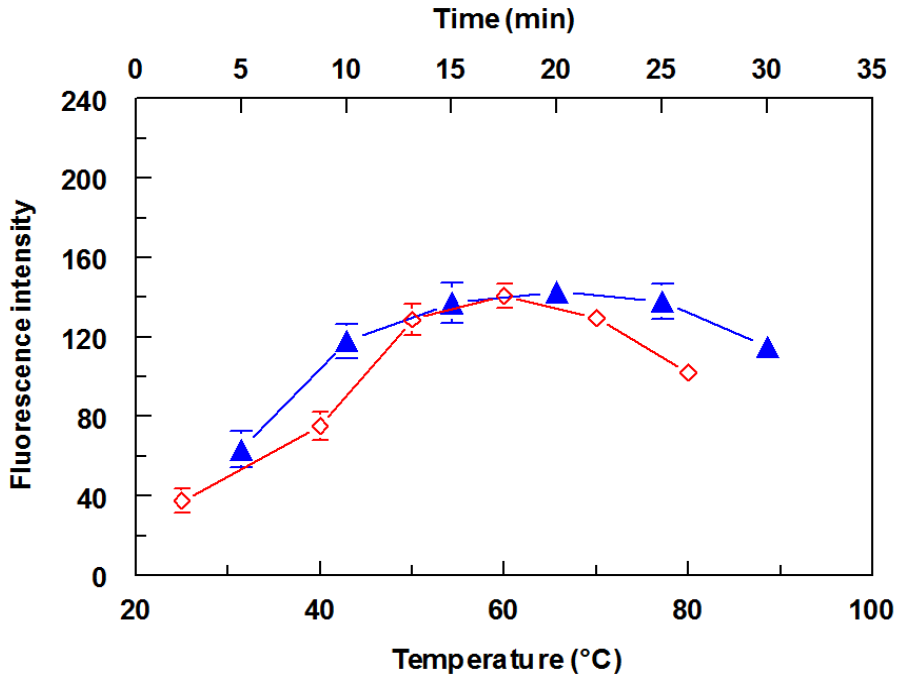

Fig. 3. Effect of temperature (◇) and time (◢) on the reaction of VRC (80 ng/mL) with NBD-Cl (0.2%, w/v).

Effect of HCl concentration

Under the above mentioned conditions, significantly high fluorescence backgrounds were also observed. This was attributed to the hydrolysis of NBD-Cl to the corresponding hydroxy derivative namely, 7-hydroxy-4-nitrobenzoxadiazole (NBD-OH) [17]. The fluorescence of NBD-OH was found to be quenched by acidifying the reaction solution [18]. Therefore, addition of HCl to the reaction mixture prior to measurement of the FI was necessary for remarkably decreasing the background fluorescence. Meanwhile, the reaction product was not affected, thus the sensitivity of the method was ultimately enhanced. The optimum concentration of HCl required for acidification was found to be 1 ml of 25 mM.

Effect of diluting solvent

In order to select the most appropriate solvent for diluting the reaction solution, different solvents were tested: water, methanol, ethanol, propanol, acetone, acetonitrile, and dioxane. The highest readings were obtained when water was used as a diluting solvent (Fig. 4). Therefore, water was used for diluting the reaction mixture in the subsequent experiments. This added an advantage to the proposed method as water is safe and not costive, rather than the toxic expensive organic solvents [19-23].

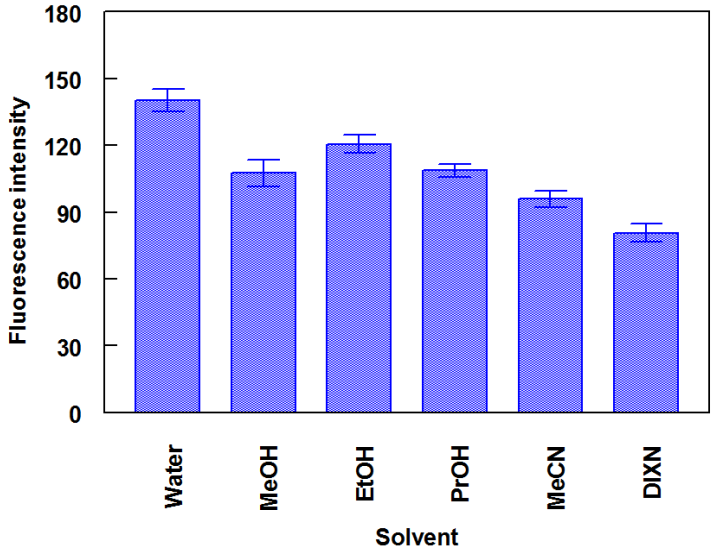

Fig. 4. Effect of diluting solvent on the fluorescence intensity of reaction product of VRC (80 pg/mL) with NBD-Cl (0.2%, w/v). Solvents were water methanol (MeOH), ethanol (EtOH), propanol (PrOH), acetonitrile (MeCN), and 1,4-dioxane (DIXN).

Stability of fluorescent derivative

The effect of time on the stability of the VRC-NBD fluorescent derivative was studied by monitoring the FI of the reaction solution (after dilution) at different time intervals. It was found that the FI values remain constant for at least 72 hour. This increased the convenience of the method as it could be applied for processing of large number of samples, and their comfortable measurements at any time within this period.

A summary for the optimization of the variables affecting the reaction of VRC with NBD-Cl is given in Table 1.

Table 1. Optimization of variables affecting the reaction of VRC with NBD-Cl

Variable	Studied range	Optimum condition
Excitation wavelength (nm)	350 – 520	470
Emission wavelength (nm)	490 – 600	550
NBD-Cl (%, w/v)	0.04 – 0.3	0.2
pH	7.5 – 10	9
Temperature (° C)	25 – 70	60
Time (min)	5 – 30	20
HCl (mM)	10 – 50	25
Solvent	Different [a]	water
Stability of VRC-NBD (hr) [b]	1 – 72	Within 72

[a] Solvents tested: water, methanol, ethanol, propanol, acetone, acetonitrile, and dioxane.
[b] The stability of the VRC-NBD was studied after dilution of the reaction solution.

Stoichiometry and mechanism of the reaction

The stoichiometry of the reaction between VRC and NBD-Cl was investigated by the limiting logarithmic method [14]. As shown in Fig. 5, two straight lines with comparable slopes were obtained indicating the 1:1 ratio for the reactions. Based on this ratio, the reaction pathway between VRC and NBD-Cl was postulated to proceed as shown in Fig. 6.

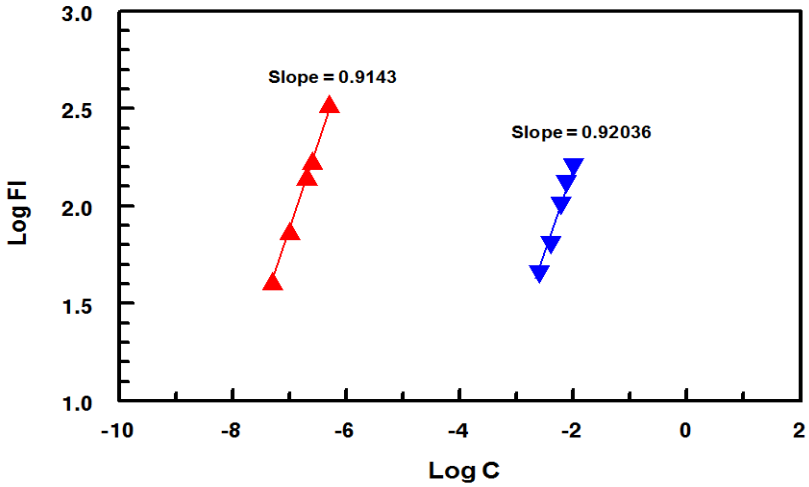

Fig. 5. Limiting logarithmic plot for molar reactivity of VRC with NBD-Cl. C and FI are the concentration and fluorescence intensity, respectively. The left-hand line (◢) was generated using varying VRC concentrations (5×10^{-8} – 5×10^{-7} M) and fixed NBD-Cl concentration (1×10^{-3} M). The right-hand line (◤) was generated using fixed VRC concentration (2×10^{-10} M) and varying NBD-Cl concentrations (2.5×10^{-3} – 1×10^{-2} M). Figures on the lines are their slopes.

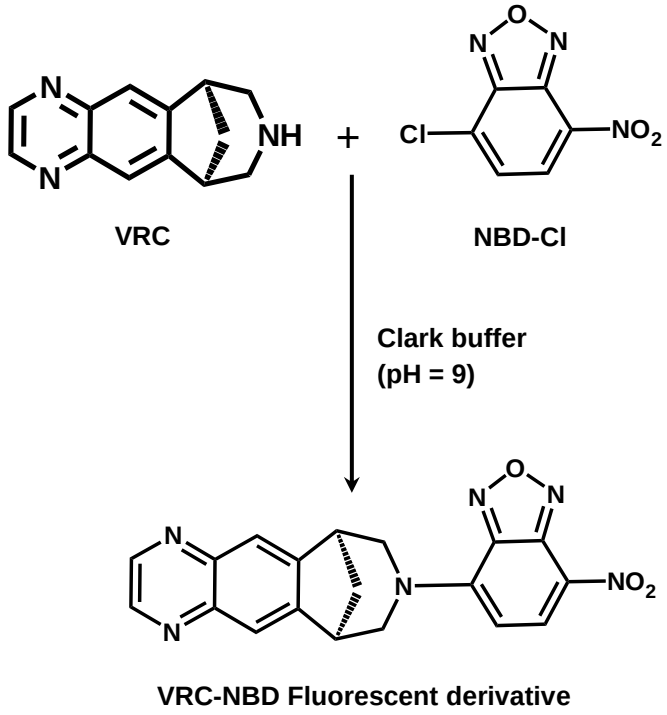

Figure 6. Scheme for the reaction pathway of VRC with NBD-Cl.

Validation of the method

Calibration and sensitivity

Under the optimum conditions (Table 1), calibration curve for the determination of VRC by its reaction with NBD-Cl was constructed by plotting the FI as a function of the corresponding VRC concentration. The regression equation for the results was: FI = 10.1254 + 1.5982 C, where FI is the fluorescence intensity, C is the concentration of VRC in ng ml^{-1}. Linear relationship with small intercept and excellent correlation coefficient (0.9993) was obtained in the range of 5–250

ml^{-1}. The LOD and LOQ were determined according to ICH guidelines for validation of analytical procedures [24]. The LOD and LOQ values were found to be 2.5 and 8.3 ml^{-1}, respectively. The parameters for the analytical performance of the proposed fluorimetric method are summarized in Table 2.

Table 2. Statistical parameters for the determination of VRC by the proposed fluorimetric method based on its reaction with NBD-Cl

Parameter	Value
λ_{ex} (nm)	470
λ_{em} (nm)	550
Linear range (ng ml^{-1})	5 - 250
Intercept	10.1254
Slope	1.5982
Correlation coefficient (r)	0.9993
LOD (ng ml^{-1})	2.5
LOQ (ng ml^{-1})	8.3

Accuracy and reproducibility

The accuracy of the proposed method was evaluated by the recovery studies for added VRC concentrations. The recovery values were 98.24 – 99.11 ± 0.88 – 1.96% (Table 3), indicating the accuracy of the proposed method. The reproducibility of the proposed method was determined by replicate analysis of 3 separate samples at three different concentration levels (low, medium and high). The method gave satisfactory results; RSD did not exceed 1.96%, indicating the good reproducibility of the method (Table 3). This precision level is adequate for the precision and routine analysis of VRC.

Table 3. Recovery and reproducibility studies for determination of VRC by the proposed fluorimetric method based on its reaction with NBD-Cl

Added VRC (ng ml^{-1})	Recovery (% ± RSD)[a]
20	99.11 ± 1.96
100	98.84 ± 1.20
200	98.24 ± 0.88

[a] Values are mean of three determinations ± RSD.

Specificity and interference liabilities

The specificity of the method was evaluated by investigating the interference liabilities from the inactive ingredients that are might be added during the formulation [22]. Samples were prepared by mixing known amount (1 mg) of VRC with 10 mg of microcrystalline cellulose, 10 mg of calcium hydrogen phosphate anhydrous, 5 mg of croscarmellose sodium, 5 mg of silica-colloidal anhydrous and 5 mg magnesium stearate. These laboratory-prepared samples were analyzed by the proposed method applying the general recommended procedure. The average recovery values were 96.05 ± 1.85–102.02 ± 1.67 % (Table 4). These data confirmed the absence of interference from any of the inactive ingredients with the determination of VRC by the proposed fluorimetric method.

Table 4. Analysis of VRC in presence of the excipients those are present in its tablets by the proposed fluorimetric method

Excipient	Recovery (% ± SD) [a]
Microcrystalline cellulose (10)[a]	96.82 ± 1.08
Crosscarmellose sodium (10)	102.02 ± 1.67
Calcium hydrogen phosphate anhydrous (5)	96.05 ± 1.85
Silica-colloidal anhydrous (5)	100.04 ± 1.91
Magnesium stearate (5)	98.82 ± 1.05

[a] Values are mean of three determinations.
[b] Figures in parenthesis are the amounts (in mg) that were added per 1 mg of VRC.

Robustness and ruggedness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that variation in the NBD-Cl concentrations (0.15–0.25%, w/v), pH (optimum ± 0.5), temperature (optimum ± 5 °C), time (optimum ± 5 min) and HCl concentration (optimum ± 5 mM) did not significantly affect the procedures; recovery values were 96.48–102.04 ± 0.82–1.85% (Table 5). These data confirmed the robustness of the proposed method.

Ruggedness was also tested by applying the method to the assay of VRC using the same operational conditions but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab and day-to-day variations were reproducible, as the RSD did not exceed 2.85%.

Table 5. Influence of small variations in the assay conditions on the analytical performance of the proposed fluorimetric method for determination of VRC using NBD-Cl reagent

Parameters	Recovery (% ± SD)[a]
Recommended conditions [b]	98.56 ± 1.46
NBD-Cl concentration (%, w/v)	
0.15	99.67 ± 1.25
0.25	100.58 ± 1.82
pH of buffer solution	
8.5	96.82 ± 1.08
9.5	101.85 ± 1.82
Temperature (° C)	
55	96.48 ± 1.05
65	98.05 ± 0.82
Reaction time (min)	
15	99.02 ± 1.85
25	102.04 ± 1.07
HCl concentration (mM)	
20	97.54 ± 0.84
30	100.24 ± 1.45

[a] Values are mean of 3 determinations ± SD.
[b] The recommended conditions were given in Table 1.

Application for analysis of VRC in tablets

It is evident from the above-mentioned results that the proposed method gave satisfactory results with VRC in bulk powder. Thus, its pharmaceutical dosage form (Champix® tablets) was subjected to the analysis of their VRC contents by the proposed method. The percentage found from the label claim was 99.58 ± 1.24%. This result was compared with those obtained by a reported method [7]; the label claim percentage was 101.31 ± 0.48%, with respect to the accuracy (by t-test), and precision (by F-test). It was found that the calculated t- and F-values (2.20 and 4.88 for t- and F-value, respectively) were lower than the tabulated ones (2.78 and 6.39 for t- and F-value, respectively). This indicated that there were no significant differences between the means and variance between the two methods in terms of the accuracy and precision.

4. Conclusions

For the first time, a highly sensitive and simple fluorimetric method for the quantitative determination of VRC has been successfully developed and validated by simple derivatization of VRC with NBD-Cl reagent, and subsequent measuring the fluorescence intensity of the highly golden yellow fluorescent reaction product. The proposed method was accurate, reproducible, and convenient for application in the analysis of tablets. By using a simple apparatus, thus the proposed method is suitable for routine analysis of VRC in quality control laboratories.

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